STUDIES ON THE DEVELOPMENT OF THE SEA URCHIN *STRONGYLOCENTROTUS DROEBACHIENSIS*. II. REGULATION OF MITOTIC SPINDLE EQUILIBRIUM BY ENVIRONMENTAL TEMPERATURE

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Modification of mitotic spindle birefringence through temperature change has proven to be a powerful tool for the investigation of spindle equilibrium and its thermodynamics. Such pioneering studies by Inoué and his collaborators (*cf.* Inoué, 1952a; Inoué and Sato, 1967) have revealed that spindle fibers are in a dynamic equilibrium with unpolymerized monomer and that shifts in this equilibrium can be correlated with spindle fiber growth and chromosome movement: a drop in temperature causes depolymerization of spindle fiber microtubules while warming causes reformation and growth. Analysis of this effect (Inoué, 1959 and 1964) indicates that the spindle monomer polymerizes with a high *positive* entropy change characteristic of hydrophobic bonding, that is, exclusion and *disordering* of many moles of bound water as one mole of monomer takes an *ordered* position in the polymer. Colchicine disrupts spindle structure, apparently through competitive binding with the free monomer (Inoué, 1952b) while D_2O enhances spindle fiber formation, presumably through some modification of water structures (Inoué, Sato, and Tucker, 1963). Thermodynamic analysis of the effects of D_2O indicates a significant change in the size of the pool of unpolymerized spindle monomer, in addition to changes in the thermodynamic parameters related to hydrogen *versus* deuterium bond energies (Carolan, Sato, and Inoué, 1966). This marked increase in pool size plus the apparent non-Newtonian mechanics needed for chromosome movement have served as focal points for criticism of the dynamic equilibrium theory (*e.g.*, Forer, 1969; McIntosh, Hepler, and Van Wie, 1969), but the mobilization of previously unavailable monomer to an active pool may have more than just secondary significance in the assembly and consequent disassembly of functioning spindle fibers and their mechanochemical role in chromosome movement.

Eggs of the sea urchin *Strongylocentrotus droebachiensis* can divide and develop to normal pluteus larvae at or below 0° C (Stephens, 1972), a temperature at which microtubules in most other organisms are non-existent. The existence, morphology, function, and control of the mitotic spindle at such low temperatures thus is of more than just academic interest. A comparison with mitosis at the higher end of the environmental temperature scale should shed some light into the dynamic nature of the spindle: is a spindle functioning at zero simply a

diminished version of its warm temperature counterpart, or does the cell have some mechanism for temperature compensation?

MATERIALS AND METHODS

Experimental animals and gametes

The sea urchin *S. droebachiensis* was obtained from the Marine Biological Laboratory Supply Department and maintained at ambient sea temperatures in the running sea water system. The animals were fed *Laminaria*. Gametes were obtained and handled as described previously (Stephens, 1972), taking all possible precautions to maintain constant temperature during shedding of gametes, washing of eggs, fertilization, and development.

Mitotic apparatus isolation

Prior to fertilization, the heavy jelly coats of the eggs were removed by careful and rapid low pH treatment (Stephens, 1972). Spindles were isolated according to modifications of the basic hexylene glycol method of Kane (1962 and 1965). The eggs were fertilized and spun down in a hand centrifuge, the sperm suspension remaining in contact with the eggs for approximately one minute at 8° C or three minutes at 0° C. The eggs were then resuspended in 1 M glycerol containing 10 mM Tris. HCl, pH 8.0 (Kane, 1970) for 15 minutes at 8° C or for 45 minutes at 0° C, in order to prevent fertilization membrane and hyaline layer formation. The eggs were transferred *via* gentle hand-centrifugation to filtered sea water of appropriate temperature and incubated in a water bath. Immediately prior to metaphase (for metaphase time at various temperatures see Stephens, 1972) the cells were washed twice with isotonic NaCl-KCl in 19:1 ratio. After one wash with 1 M hexylene glycol buffered at pH 6.4 with 10 M phosphate, the cells were lysed in fresh hexylene glycol medium by vortex-mixing. In all of the above washes, the reagent volumes were ten times the egg volume; one ml of eggs was typically used. The temperature of the NaCl-KCl and hexylene glycol medium was determined by the experimental design. Immediately upon release of the mitotic apparatuses, the solution was chilled in ice. The spindles were then sedimented at $2000 \times g$ for 2 minutes in a clinical centrifuge and washed three times *via* centrifugation with 5 ml of ice-cold isolation medium. The spindles were resuspended in about 0.1 ml of isolation medium for observation.

Microscopy

Observations in polarized light were made within a Leitz Ortholux Pol microscope equipped with a Xenon light source, Baird-Atomic 546 nm interference filter, heat filter, and selected strain-free optics. A Brace-Koehler compensator of 18 nm retardation was used for measurement of birefringence. Photographs were taken with Kodak Panatomic-X film developed in Diafine developer. Phase-contrast observations were made with Zeiss optics and photographed on Panatomic-X film developed in Kodak Microdol-X. When both phase-contrast and polarization micrographs were necessary, a phase annulus was placed in the position normally occupied by the lamp iris in the base of the Ortholux microscope and a pre-

centered phase-contrast objective was swung into place by means of a pre-centered turret. It was thus possible to switch from polarized light to phase-contrast and back without moving the specimen or removing the strain-free polarizing condenser. The analyzer, of course, was removed for phase-contrast observation, but (given a sufficiently strain-free phase objective) the analyzer could be used to obtain a combined polarization-phase-contrast image, useful in visually relating mass and birefringence.

Cells at the appropriate stage were placed on a water-cooled temperature controlled slide, the temperature of the specimen being taken as the average of the influx and efflux temperatures. Coverslip spacers, 0.18 mm thick, were used to prevent compression; when compression was desired, no spacer was used and the fluid was drawn from beneath the single coverslip with filter paper until the desired degree of compression was obtained.

Gel electrophoresis

Mitotic apparatus preparations were analyzed electrophoretically using 5% polyacrylamide gels containing sodium dodecyl sulfate (SDS) by methods devised by Shapiro, Vinuela, and Maizel (1966) and modified by Weber and Osborn (1970). The gels were stained with Fast Green (Gorovsky, Carlson, and Rosenbaum, 1970) by appropriately replacing the Coomassie Blue dye in the Weber-Osborn formulation, and then quantitated by densitometry. The position of tubulin in the mitotic apparatus preparations was established by a parallel gel containing the pure protein obtained from sperm flagellar outer fibers (Stephens, 1970).

Tubulin synthesis

Tubulin synthesis at $0°$ C and at $8°$ C was monitored at ten successive intervals during the first division by pulse-labeling 0.2 ml of eggs with 5 μc of ^{14}C-leucine in 2.0 ml of sea water, separating the tubulin from 1 mg of total cell homogenate with SDS-polyacrylamide gel electrophoresis, and then either eluting the protein from the gel and counting by scintillation methods or by direct autoradiography of the longitudinally-sliced gel (Fairbanks, Levinthal, and Reeder, 1965). Full details of these methods are outlined in another paper of this series (Stephens, in preparation).

Chemicals

Uniformly-labeled ^{14}C-leucine, with a specific activity of 312 mC/mmole, was obtained from Schwarz/Mann, Orangeburg, New Jersey. Colchicine was obtained from City Chemical Corporation, New York, New York.

RESULTS

Observation and isolation of the first division mitotic spindle at $8°$ C and $0°$ C or after temperature jumps to $20°$ C

Two types of determinations were made. Cells were fertilized and grown at either $0°$ C or at $8°$ C until metaphase; the spindle birefringence was measured,

the cells were brought to 20° C, and the birefringence was remeasured. As an alternative to these *in vivo* determinations, the mitotic apparatus was isolated at either 0° C, 8° C, or at 20° C after prior growth to metaphase at either of the former temperatures; the birefringence of the spindle isolate was then determined *in vitro*. No significant birefringence differences were noted between the *in vivo* and the *in vitro* measurements.
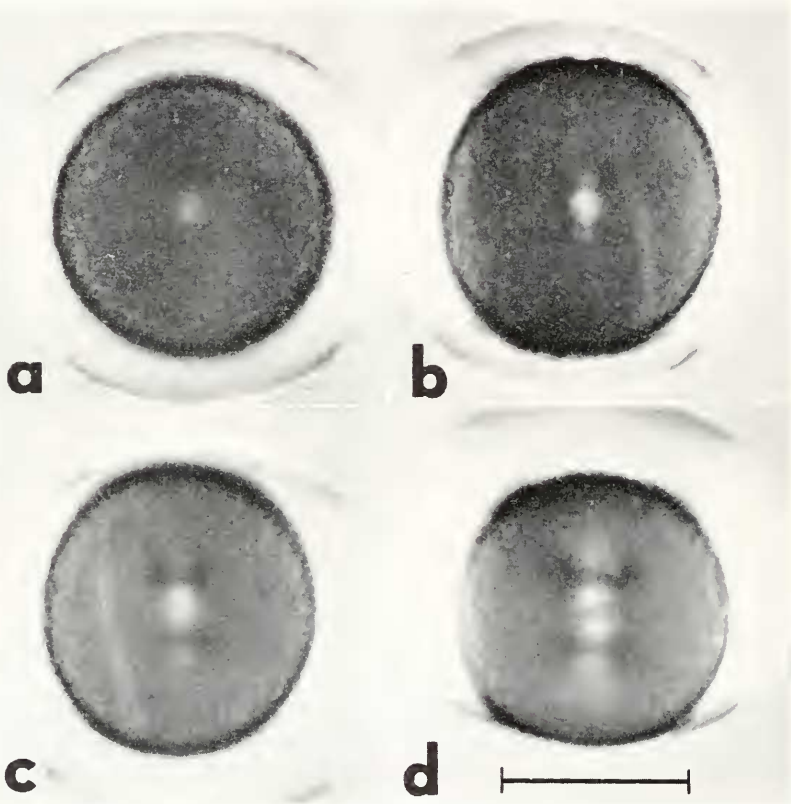

FIGURE 1. Eggs at metaphase grown at two specific environmental temperatures and transferred to 20° C, (a)—cell fertilized and grown at 0° C, retardation = 1 nm; (b)—same as (a) but brought to 20° C at metaphase, retardation = 2.5 nm; (c)—cell fertilized and grown at 8° C, retardation = 2.7 nm; (d)—same as (c) but brought to 20° C at metaphase, retardation = 4.5 nm, scale = 100 μ.

At 8° C the mitotic spindle *in vivo* was of the normal amphiastral type, measuring 30 μ from pole to pole, and having a metaphase retardation of 2.75 ± 0.25 nm, measured in the central spindle (Fig. 1c). When these cells were brought to 20° C, the birefringence increased markedly, reaching a maximum retardation of 4.50 ± 0.25 nm within two minutes. Large asters formed and the spindle "enlarged" somewhat, measuring 40–50 μ from pole to pole (Fig. 1d). Transfer of cells from 8° C to 0° C caused no obvious morphological changes, but reduced

the retardation of the central spindle region to 1.5 ± 0.2 nm. Asters were still present but very weakly birefringent. When spindles were isolated at 8° C, and at 20° C after growth to metaphase at 8° C, essentially identical results were obtained but the differences in both morphology and birefringence were substantially more obvious (Fig. 2b and 2d compared with Fig. 1c and 1d).
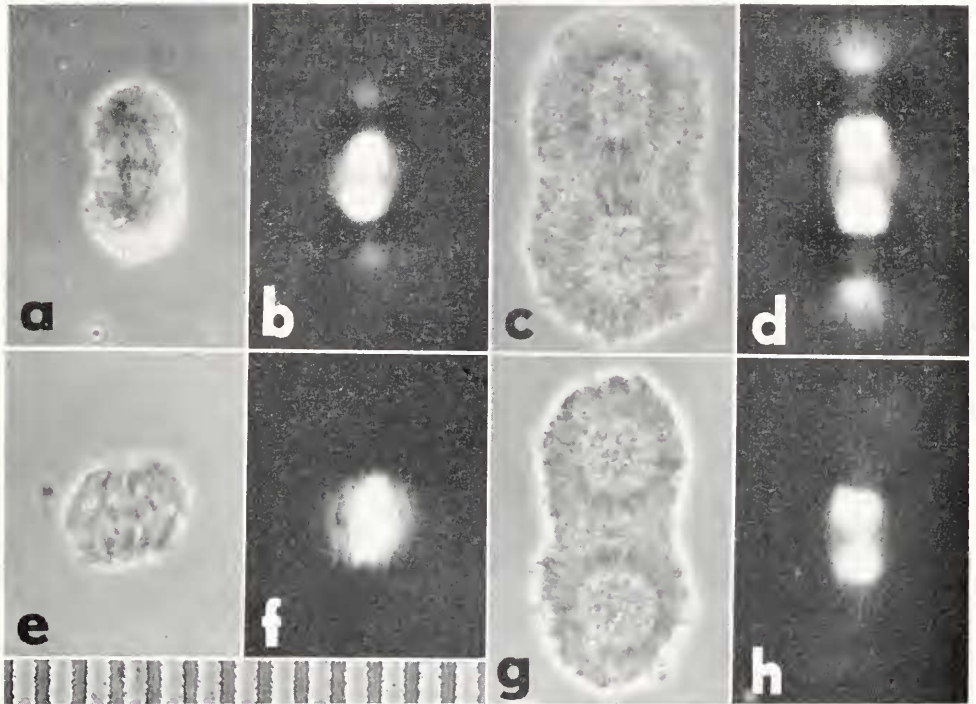

FIGURE 2. Spindles isolated at metaphase, grown at two specific environmental temperatures and transferred to 20° C, (a)—cell fertilized and grown at 8° C, spindle isolated at metaphase, phase-contrast optics; (b)—same as (a), polarization optics; (c)—cell fertilized and grown at 8° C, but spindle isolated after transfer to 20° C, phase-contrast optics; (d)—same as (c), polarization optics; (e)—cell fertilized and grown at 0° C, spindle isolated at metaphase, phase-contrast optics; (f)—same as (e), polarization optics; (g) cell fertilized and grown at 0° C, but spindle isolated after transfer to 20° C, phase-contrast optics; (h)—same as (g), polarization optics; Scale intervals = 10 μ.

At 0° C no "spindle" as such was observed. Rather, an apparent *uniaxial sphere* with a diameter of about 30 μ and a retardation of 0.95 ± 0.2 nm was typically observed; no distinct poles or asters were evident (Fig. 1a). Transfer of these cells to 20° C resulted in the enhancement of central spindle birefringence to a value of 2.3 ± 0.25 nm, a pole to pole distance of 40–50 μ, but only barely perceptible growth of weakly birefringent asters (Fig. 1b). Cells grown at 0° C and brought to 8° C had a central spindle retardation of 1.5 ± 0.2 nm. Isolation of the 0° C spindle revealed that the uniaxial sphere seen *in vivo* was a totally anastral spindle (Fig. 2e and 2f) while the spindle of cells grown at 0° C and brought to 20° C consisted primarily of birefringent chromosomal and continuous

spindle fibers with an astral region of considerable mass but nearly immeasurable birefringence (Fig. 2g and 2h).

Execution of the "growth phase" of the above experiment at intermediate temperatures resulted in the production of intermediate spindles with respect to both birefringence and morphology not only at the temperature of growth but also after the temperature jump.

These data are consistent with the hypothesis that there is more spindle fiber monomer available in cells grown at 8° C than 0° C since the birefringence of a spindle grown at 8° C and transferred to 20° C is twice that for a spindle grown at 0° C and similarly transferred or, alternatively, the birefringence of a spindle grown at 8° C and brought to 0° C is at least 50% higher than that of a spindle grown and observed at 0° C. This argument makes use of only the central spindle birefringence; the amphiastral nature of the 8° C spindles provides even

TABLE 1

Birefringence and tubulin content data for spindles at various growth and isolation temperatures

| Temperature | | Birefringence nm retardation* | Relative tubulin† | Relative 22S† |
Growth	Isolation			
0°	0°	0.95	0.3	0.6
0°	8°	1.50		
0°	20°	2.30	0.9	3.1
8°	0°	1.50		
8°	8°	2.75	1.0	1.0
8°	20°	4.50	1.9	4.2

* Measured in the central spindle: Retardation = Compensator Retardation $\times$ sin 2θ, where θ = difference between maximum background compensation and maximum spindle compensation angles, with specimen at 45°. Error is $\pm$ 5%.

† Relative to that obtained from 8° C spindles isolated at 8° C.; determined microdensitometrically from the tubulin or 22S bands obtained from the SDS-acrylamide gel electrophoresis of spindles from equal numbers of eggs.

more evidence for this conclusion. Table 1 summarizes the above birefringence data and includes an estimate of the relative amount of tubulin and 22S protein obtained from the spindles of an equal number of eggs grown at 0° C and 8° C, or grown at these temperatures and transferred to 20° C for isolation. Figure 3 illustrates a typical SDS-acrylamide gel electrophoretic pattern obtained from *S. droebachiensis* spindle isolates, accompanied by parallel gels of a whole egg homogenate and of sperm flagella axonemes. Only the tubulin fraction was found to be consistently proportional to the birefringence of the spindle. Admittedly, this correlation of birefringence with tubulin content represents only a first approximation since no correction has been applied here for geometrical differences in the central spindle regions of the various spindle preparations. The other components, however, particularly the 22S and 2.5S proteins (Stephens, 1967), varied directly with the spindle volume and inversely with the degree of washing. The stoichiometry of mitotic apparatus components and their possible role in mitosis

are currently being investigated further, but the results of this present work indicate that the majority of them arise by a "sponge" effect due to trapping of cytoplasmic components. This is quite consistent with the recent finding that the 22S and 2.5S protein "mitotic" components are constituents of the yolk granules or other particulate fractions and not of the spindle fibers themselves (Burns and Kane, 1971).
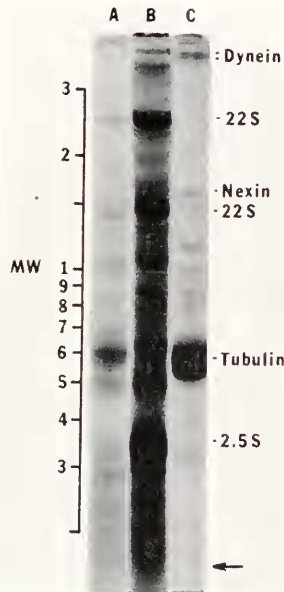

FIGURE 3. Comparative SDS-polyacrylamide gels of mitotic apparatuses isolated at 8° C, (A), whole egg homogenate (B), and sperm flagellar axonemes (C); flagellar components from Stephens (1971); 22S and 2.5S components from calibration with yolk particulate fraction (cf. Burns and Kane, 1971); lower molecular weight (MW) cycle = 20,000 to 100,000; upper cycle = 100,000 to 400,000 (not accurate above 250,000 and below 30,000 due to deviations characteristic of 5% gel systems). No dynein is observed in the mitotic apparatus isolates, although a *single* dynein band is apparent in the whole egg homogenate, corresponding in mobility to the higher molecular weight dynein subunit in flagella. Arrow indicates the position of the original marker-dye front.

Electrophoretic separation and subsequent counting of tubulin from homogenates of eggs pulse-labeled throughout the first division showed no significant difference in the amount of total tubulin synthesized at any given point in the cell cycle at either 0° C or at 8° C; the overall pattern of protein synthesis was also identical in both cases.

Second division metaphase at a new environmental temperature

In the next division of the cell, is the amount of spindle monomer available for use influenced by temperature conditions during the first division?

When cells were grown at 0° C through cytokinesis and then transferred to 8° C, mitosis proceeded precisely according to an 8° C timetable for the second

division (*cf.* Stephens, 1972). Similarly, cells grown at 8° C and transferred to 0° C just following cytokinesis behaved as typical 0° C cells in second division. Observation of the second division spindle under these respective conditions showed no significant deviations in spindle fiber birefringence or overall morphology when compared to spindles from eggs grown at a single temperature. Thus, temperature conditions of the first division have no influence on spindle birefringence and morphology, or on the time course of the second division. Further division results in somewhat smaller spindles and reduced asters, making any later comparisons difficult.

The influence of temperature changes during the first division upon the character of the metaphase spindle

When does the cell "mobilize" the prescribed amount of spindle monomer for its charcteristic spindle?

Eggs from the same female were fertilized and grown at either 0° C or 8° C and then transferred to the opposite temperature at various points in the pre-metaphase cell cycle. The type of spindle that developed was judged by both size and birefringence. In the following description, a "0° C spindle" will refer to a uniaxial sphere with a retardation of about 1 nm while an "8° C spindle" will refer to an amphiastral spindle with approximately 3 nm retardation (for example, Fig. 2B *versus* Fig. 2F).

At any point up to and including nuclear membrane breakdown, cells fertilized and initially grown at 8° C and then transferred to 0° C developed typical 0° C spindles; transfer during early prophase brought about intermediate types of spindles having a retardation of about 2 nm and developing small asters. Few of these cells underwent cytokinesis according to the 0° C timetable when the transfer took place at or after nuclear membrane breakdown; rather, they underwent a highly asynchronous, multipolar second division.

Cells fertilized and grown at 0° C and then transferred to 8° C at any point up to and including nuclear membrane breakdown produced spindles characteristic of cells grown entirely at 8° C. The remainder of the division cycle proceeded according to an 8° C time scale and normal cytokinesis took place. Transfer during early prophase, as in the reverse transfer cited above, resulted in the formation of intermediate spindle types.

When cells are properly flattened, nuclear membrane breakdown and early prophase can be observed with great precision through optical sectioning. From the foregoing observations, the "switch point" in determining the type of spindle that will form appears to be early in prophase, when the asters are actually in the process of forming. Differences in synchrony and temperature variations during observation make it impossible to be more exact.

Discussion

Implications for mitotic equilibrium

A number of new facts can be immediately pointed out as being revelant to mitosis in its broadest sense:

1. *Spindle fibers can and do exist at 0° C* as evidenced by a typical 0° C spindle (Fig. 2e and 2f) or by the fact that an 8° C spindle brought to 0° C loses less than half of its birefringence (Table I).

2. *Functional spindles of the same division cycle in the same organism can be either amphiastral or anastral* (Fig. 2a *versus* 2e); temperature-induced growth of the anastral type results principally in the increase of chromosomal and continuous fiber birefringence (Fig. 2h).

3. Substantially more spindle monomer is available at 8° C than at 0° C, whether judged by spindle birefringence or tubulin content (Table I), demonstrating that *monomer pool size can be naturally varied.*

4. *The monomer is apparently made available at one discreet point in the cell cycle,* with the amount being determined by the temperature during *early prophase.*

5. *No temperature compensation appears to exist;* rather, the process of activation or mobilization during early prophase behaves like a biological process whose temperature coefficient differs markedly from the average of the cell as a whole.

The ability of *S. droebachiensis* to form spindle fibers at such extremely low temperatures very likely indicates the evolution of a tubulin whose subtleties in primary structure permit a substantially stronger degree of association than that from animals living at higher temperatures. In this regard it might be pointed out that isolated spindles of *S. droebachiensis* retain their birefringence for significantly longer periods of time when stored at room temperature in isolation medium than do those from *Arbacia punctulata,* a sea urchin having a 15° C higher temperature range. Kane and Forer (1965) report a retardation of 3–4 nm for spindles isolated at room temperature from the latter urchin; approximately half of the birefringence is lost from such spindles in one hour at room temperature. Spindles from *S. droebachiensis,* however, have this same retardation when isolated at 8° C and they lose birefringence at about 25% per hour at room temperature. The response to colchicine is likewise somewhat slower; concentrations roughly twice those used by Inoué (1952b) were needed to decrease birefringence in *S. droebachiensis* at a rate comparable to those which he achieved for *Chaetopterus.* Confirmation of the hypothesis of a more strongly-associating mitotic tubulin must await *in vitro* comparison of *S. droebachiensis* tubulin with that from another species whose spindle fibers are substantially more cold labile.

The functional existence of an anastral spindle in the form of a uniaxial sphere at 0° C would indicate that the astral regions of the more typical sea urchin spindle would have little function in mitotic movement *per se.* Inoué (1964) has observed that birefringence is strongest near poles and centromeres, the "orienting centers" of his dynamic equilibrium theory. Brief reference to Figure 2 illustrates that the birefringence is strongest between the chromosomes and the "centrosphere," with the "0° C to 20° C" spindle (the condition with apparently the least amount of polymerizable spindle fiber material) devoting essentially all of its oriented material to the formation of chromosomal and continuous spindle fibers (Fig. 2h). With presumably a great deal more available spindle precursor, the "8° C to 20° C" spindle apparently can afford to form large asters (Fig. 2d). If spindle material is conserved during development, and not appreciably synthesized, spindles should get smaller with each successive division; Harris (1962) not only notes

that this does happen but she also observes that spindles become anastral. Harris speculates that perhaps the possession of asters during the first several divisions may be a specialization used by large cells to guarantee equal cytokinesis but the fact that the totally anastral 0° C first division spindle in *S. droebachiensis* (an egg 160 μ in diameter) partitions the egg quite equally would argue against this point. A striking feature of the temperature-induced "giant" spindles of *S. droebachiensis* is the huge "centrosphere," strikingly visible in the phase-contrast micrographs (Fig. 2c and 2g); polarized light images indicate no clear-cut single "center" as is so apparent in the untreated spindles at 8° C (compare Fig. 2d with 2b). Ultrastructural studies of high and low-temperature spindles, with particular emphasis on the centrosphere region are currently underway (Stephens, in preparation).

The study by Carolan, Sato, and Inoué (1966) of the effects of D_2O on metaphase-arrested *Pectinaria gouldii* oocytes indicated that this agent reversibly increases the amount of spindle precursor available for polymerization, in addition to modifying the thermodynamic parameters of association. Though not so stated, the mobilization of polymerizable spindle fiber material prior to metaphase and the reverse process thereafter could provide the motile force needed by the dynamic equilibrium theory. Indeed, this change in "pool" is necessary for the theory since it simultaneously assumes that the equilibrium constant is truly constant and that at any given moment there is an upper limit to the amount of available spindle fiber precursor. Otherwise, how can the equilibrium be dynamic? Arguments can be made that D_2O effects a multitude of things in the cell, that arrested metaphase is a unique situation, and that response to any agent is dependent upon the stage of mitosis, but the evidence presented here for environmentally-controlled or modified spindles is not subject to these arguments. The overall pool size is clearly different in the two temperature cases.

The fact that some temperature-dependent process during prophase (by definition, the time during which the spindle is assembled) determined how much total spindle material is mobilized would point to the possibility that there exists, in the cell, sites possessing this same potential which may be active in either a forward or reverse direction during mitotic movement. Thus some simple biochemical process may locally activate or deactivate spindle material during the mitotic cycle, bringing about the localized polymerization-depolymerization postulated in the dynamic equilibrium theory (Inoué, 1964). It may be significant that during mid-to-late anaphase, in spite of the presence of "orienting centers" at the poles and kinetochores, increase in temperature causes little change in birefringence in either 0° or 8° C cells, impling an inactivation of previously-available spindle fiber monomer.

Over an organism's normal temperature range, the developmental processes generally follow a rate *versus* temperature relationship characteristic of the sum total of enzyme rates in the cell. Early studies of cleavage rates (Hoadley and Brill, 1937), first division events (Fry, 1936), or respiration during embryogenesis (Tyler, 1942) at various temperatures within the viable range indicated that the log of the respective rates varied inversely with temperature. The whole pattern of early development in *S. droebachiensis* follows the same proportionality sequence regardless of temperature; only the relative rates vary (Stephens, 1972).

It is in this one unique case—spindle formation—where a developmental event apparently has a substantially different activation energy from the "average" process in the cell and where the product of this developmental event, the spindle fiber material, itself undergoes a temperature-dependent polymerization process. Thus, even though the overall pattern of protein synthesis and the proportionality of the timetable of mitotic events is independent of temperature, this one event can be "dissected" from the rest.

Some thoughts on the mechanism of mitosis

The purpose of this paper is to present data, not dogma. But since the interpretation of this data must eventually be evaluated in the context of existing theories of mitosis, comment on its consistency with such concepts seems in order.

It has already been pointed out that the demonstration of a natural change in pool size and the consequent implication of some prophase event mobilizing polymerizable spindle fiber material are findings which support and strengthen the dynamic equilibrium theory of Inoué. That a similar mobilization or de-mobilization may take place locally during anaphase movement is admittedly speculation, but it is an idea that is central to the Inoué mechanism. In addition, the results presented here demonstrate that the amount of tubulin obtained from a spindle is directly proportional to the birefringence, while other components vary with spindle size or degree of washing. This observation should lay to rest the criticism that birefringence is not necessarily a measure of oriented microtubule material (cf. discussion in Forer, 1969). The present experiments do not contribute any further insight into the mechanism of force production by polymerization and depolymerization of microtubules, a seeming violation of Newtonian physics. In this regard, however, Inoué, (1952b) clearly demonstrated that, in Chaetopterus, when one pole is anchored to the cell cortex, application of colchicine in low concentration brought about simultaneous diminution of birefringence and chromosome movement toward the cell cortex, indicating the depolymerization can result in a "pulling" force. Induced growth of spindles, whether metaphase-arrested or not, either by heat or D_2O, results in a marked increase in interpolar distance (Inoué and Sato, 1967; this report, Fig. 2a versus 2c), so there is little doubt that increased polymerization can result in a "pushing" force. It is still not clear, however, exactly how this force can be generated. Perhaps some cyclic anchoring mechanism involving "matrix" material around the tubules is involved or perhaps local conformational changes within the tubule substructure (e.g., Thomas, 1970) may allow addition or removal of spindle monomers in the center of a spindle fiber without affecting its integrity, a notion which at the time that it was proposed (Inoué, 1964) was thought to be implausible by many.

Various sliding filament mechanisms have been proposed for chromosome movement (e.g., Bělař, 1929; Bajer, 1968; Subirana, 1968; McIntosh, Hepler, and Van Wie, 1969). The mechanism of McIntosh et al. (1969) is a variation on a theme by Huxley (1969) involving polarized chromosomal and continuous spindle fibers with appropriately oriented cross-bridges; the others are less specific and hence far less testable. Mechanically and mechanistically, sliding filaments offer an attractive hypothesis, but numerous questions must still be answered.

In order to slide over one another, filaments must be close enough to do so and must be attached to the proper places; spatial arrangement and microtubule continuity are major unresolved problems at this point (see through discussion in Nicklas, 1971). Even where appropriately connected and arranged microtubules are close enough to interact, they must have something to interact with; both Wilson (1969) and McIntosh, Hepler, and Cleland (1971) present evidence for specific bridges, but more conclusive evidence is needed on this point. Where interaction is postulated to be with adjacent cytoplasmic material (Subirana, 1969), where is and what is this material? What prevents sliding of filaments before metaphase and what controls their simultaneous movement and disappearance during anaphase? Poleward particle movement (Bajer, 1958) or poleward movement of a UV-irradiated spot (Forer, 1965), both prior to metaphase and both with rates comparable to anaphase movement, indicate that a great deal might be taking place before the actual movement of chromosomes, a time when sliding filaments should be inactive. Under any sliding mechanism, how do spindles that *eliminate* chromosomes function (Metz, 1938)? Perhaps the chromosome should not be treated simply as a passenger!

Regarding the temperature-equilibrium observations made here, one might ask the question of how a spindle with sliding filaments can still function quite normally with half of its usual filament material? Intuitively, one might expect cells to have evolved a true temperature compensation mechanism wherein *more* spindle material is mobilized at lower temperatures to offset the depolymerizing effect of such temperatures. It is also not easy to visualize how mitotic movement *via* an active sliding process would be consistent with the observations of chromosome movement by simple depolymerization (Inoué, 1952b) or the change in interpolar distance by heat or D_2O treatment (Inoué, and Sato, 1967) discussed above.

Probably the only indisputable conclusion that can be drawn from the foregoing discussion is that a great deal more work on this problem is warranted.

On the possible nature of the mobilization process

It is generally agreed that the major components of the mitotic apparatus (in eggs at least) pre-exist in the cell, to be utilized at prophase (Mazia, 1961), and hence the idea of a mobilization of spindle fiber material is clearly not new. Its final proof must still await a demonstration of differences between polymerized spindle material and that from the postulated inactive pool. Several plausible biochemical mechanisms come immediately to mind. Guanosine triphosphate is a necessary cofactor for polymerization of flagellar tubulin (Stephens, 1968 and 1971); possibly the addition of the nucleotide to the tubulin or the phosphorylation of bound guanosine diphosphate is the activation step. Cyclic changes in -SH content of both the whole cell and the mitotic apparatus are well documented (Mazia, 1961) and experimental modification of -SH balance can cause reversible depolymerization of the spindle (Stephens, Inoué, and Clark, 1966). Either the formation or the breakage of intramolecular disulfide bonds may cause sufficient conformational change to promote markedly different association properties for the oxidized or reduced form. Amination, glucosylation, phosphorylation or other post-synthesis modification of amino acid side chains would offer another

possible activating step. Peptide cleavage, as in the fibrinogen-fibrin transformation, is an unlikely possibility since such a process during mitosis would very likely need to be reversible. The possibility exists that other proteins besides tubulin "copolymerize" in the microtubule; the presence or absence of these postulated cofactors might in turn determine microtubule polymerization initiation and kinetics. Exactly what this cellular control process involves thus poses a crucial problem; its nature is fundamental to the whole question of microtubule polymerization and function, and perhaps to mitosis itself.

SUMMARY

1. The assembly and function of the mitotic apparatus in first division eggs of the sea urchin *Strongylocentrotus droebachiensis* were studied at 0° C and at 8° C by polarization microscopy *in vivo* and after isolation in hexylene glycol at controlled pH.

2. No differences in the amounts of total tubulin synthesized over comparable periods of the cell cycle were observed.

3. Mitotic apparatuses from a cell grown at 0° C are anastral, while those grown at 8° C are amphiastral.

4. At a 0° C growth temperature only about one-half of the spindle fiber monomer is available for polymerization as at 8° C, indicating a natural variation in pool size.

5. The amount of spindle monomer made available to the usable pool is specified only by the temperature during early prophase, with temperature prehistory having no effect.

6. The temperature coefficient for this apparent activation differs markedly from that of the mitotic process as a whole.

7. The amount of tubulin obtained from an isolated mitotic apparatus, as determined by electrophoretic separation of tubulin on SDS-acrylamide gels, is directly proportional to its measured retardation, implying that birefringence is a true measure of oriented microtubules.

8. These results are compatible with the mitotic dynamic equilibrium theory of Inoué and complement this theory by providing evidence for natural variation in pool size (and thus for the potential local mobilization of monomer during anaphase movement) and by providing a direct correlation between birefringence and tubulin content of spindles.

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